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November 10, 1999

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Assistant Commissioner for Patents  
Washington, D.C. 20231

**Box Patent Application**

Re: New U.S. Non-Provisional Utility Patent Application  
Appl. No. To be assigned; Filed: Herewith  
For: **Recovering Metals from Soil**  
Inventors: Chaney *et al.*  
Our Ref: 1797.0090005/RWE/KKV

Sir:

The following documents are forwarded herewith for appropriate action by the U.S. Patent and Trademark Office:

1. U.S. Utility Patent Application entitled:

**Recovering Metals from Soil**

and naming as inventors:

Rufus L. Chaney  
Jayce Scott Angle  
Yin-Ming Li  
Alan J.M. Baker

the application comprising:

The PTO did not receive the following  
listed item(s) 1 post card.

JC525 U.S. PTO  
09/437607  
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Assistant Commissioner for Patents

November 10, 1999

Page 2

a specification containing:

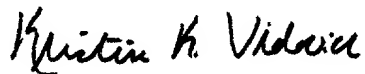
- (i) 30 pages of description prior to the claims;
  - (ii) 7 pages of claims (47 claims);
  - (iii) a one (1) page abstract;
- 2. USPTO Utility Patent Application Transmittal Form PTO/SB/05;
  - 3. 37 C.F.R. § 1.136(a)(3) Authorization to Treat a Reply As Incorporating An Extension of Time (*in duplicate*); and
  - 4. Two (2) return postcards.

It is respectfully requested that, of the two attached postcards, one be stamped with the filing date of these documents and returned to our courier, and the other, prepaid postcard, be stamped with the filing date and unofficial application number and returned as soon as possible.

This patent application is being submitted under 37 C.F.R. § 1.53(b) without Declaration and without filing fee.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



Kristin K. Vidovich  
Attorney for Applicants  
Registration No. 41,448

KKV/pcd  
Encls.

09437607-11099

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Approved for use through 09/30/2000. OMB 0651-0032

Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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|  |  |                             |                      |
|--|--|-----------------------------|----------------------|
| <p><b>UTILITY PATENT APPLICATION TRANSMITTAL</b></p> <p><i>(Only for new nonprovisional applications under 37 CFR § 1.53(b))</i></p> | Attorney Docket No.                      |                             | 1797.0090005/RWE/KKV |
|  | First Inventor or Application Identifier |                             | Rufus L. Chaney      |
|  | Title                                    | Recovering Metals from Soil |                      |
|  | Express Mail Label No.                   |                             |                      |

| <b>APPLICATION ELEMENTS</b><br><i>See MPEP chapter 600 concerning utility patent application contents.</i>  | <b>ASSISTANT COMMISSIONER for Patents</b><br><b>Box Patent Application</b><br><b>Washington, DC 20231</b>  |
|---|--|
| <p>1. <input type="checkbox"/> * Fee Transmittal Form (e.g., PTO/SB/17)<br/> <i>(Submit an original, and a duplicate for fee processing)</i></p> <p>2. <input checked="" type="checkbox"/> <b>Specification</b> [Total Pages <u>38</u> ]<br/> <i>(preferred arrangement set forth below)</i></p> <ul style="list-style-type: none"> <li>- Descriptive title of the invention</li> <li>- Cross References to Related Applications</li> <li>- Statement Regarding Fed sponsored R &amp; D</li> <li>- Reference to Microfiche Appendix</li> <li>- Background of the invention</li> <li>- Brief Summary of the invention</li> <li>- Brief Description of the Drawings <i>(if filed)</i></li> <li>- Detailed Description</li> <li>- Claim(s)</li> <li>- Abstract of the Disclosure</li> </ul> <p>3. <input type="checkbox"/> Drawing(s) (35 U.S.C. 113) [Total Sheets <u>    </u> ]</p> <p>4. <input type="checkbox"/> Oath or Declaration [Total Pages <u>    </u> ]</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> Newly executed (original or copy)</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> Copy from a prior application (37 CFR 1.63(d)) <i>(for continuation/divisional with Box 17 completed)</i><br/> <b>[Note Box 5 below]</b></p> <p style="margin-left: 40px;">i. <input type="checkbox"/> <b>DELETION OF INVENTOR(S)</b><br/> Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR §§ 1.63(d)(2) and 1.33(b).</p> <p>5. <input type="checkbox"/> Incorporation By Reference <i>(useable if Box 4b is checked)</i><br/> The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.</p> | <p>6. <input type="checkbox"/> Microfiche Computer Program <i>(Appendix)</i></p> <p>7. Nucleotide and/or Amino Acid Sequence Submission <i>(if applicable, all necessary)</i></p> <p style="margin-left: 20px;">a. <input type="checkbox"/> Computer Readable Copy</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> Paper Copy (identical to computer copy)</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> Statement verifying identity of above copies</p>  |
| <b>ACCOMPANYING APPLICATION PARTS</b>   |  |
| <p>17. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:</p> <p style="margin-left: 20px;"> <input type="checkbox"/> Continuation      <input type="checkbox"/> Divisional      <input checked="" type="checkbox"/> Continuation-in-Part (CIP) of prior application No: <u>09/386,373</u> </p> <p style="margin-left: 20px;"> Prior application information: Examiner _____ </p>   | <p>8. <input type="checkbox"/> Assignment Papers (cover sheet &amp; document(s))</p> <p>9. <input type="checkbox"/> 37 CFR 3.73(b) Statement <input type="checkbox"/> Power of Attorney<br/> <i>(when there is an assignee)</i></p> <p>10. <input type="checkbox"/> English Translation Document <i>(if applicable)</i></p> <p>11. <input type="checkbox"/> Information Disclosure Statement (IDS)/PTO-1449 <input type="checkbox"/> Copies of IDS Citations</p> <p>12. <input type="checkbox"/> Preliminary Amendment</p> <p>13. <input checked="" type="checkbox"/> Two Return Receipt Postcards (MPEP 503)<br/> <i>(Should be specifically itemized)</i></p> <p>14. <input type="checkbox"/> *Small Entity Statement(s) <input type="checkbox"/> Statement filed in prior application, Status still proper and desired<br/> <i>(PTO/SB/09-12)</i></p> <p>15. <input type="checkbox"/> Certified Copy of Priority Document(s)<br/> <i>(if foreign priority is claimed)</i></p> <p>16. <input checked="" type="checkbox"/> Other: 37 C.F.R. § 1.136(a)(3) Authorization</p> <p style="margin-left: 20px;"><input type="checkbox"/> Other:</p> <p style="font-size: small;">*NOTE FOR ITEMS 1 &amp; 14. IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).</p> |
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| NAME  | STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.<br>Attorneys at Law |  |                |  |                |
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|                   |                     |                                   |               |
|-------------------|---------------------|-----------------------------------|---------------|
| NAME (Print/Type) | Kristin K. Vidovich | Registration No. (Attorney/Agent) | 41,448        |
| SIGNATURE         | Kristin K. Vidovich | Date                              | Nov. 10, 1999 |

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# Recovering Metals from Soil

## *Background of the Invention*

### *Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development*

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

### *Cross-Reference to Related Applications*

This application is a continuation-in-part of U.S. Application No. 09/386,373, filed August 31, 1999, which is a continuation-in-part of U.S. Application No. 08/879,813, filed June 20, 1997, now U.S. Patent No. 5,944,872, which is a continuation of U.S. 08/470,440, filed June 6, 1995, now U.S. Patent No. 5,711,784, and this application claims priority to U.S. Provisional Application Nos. 60/109,443, filed November 23, 1998, and 60/107,797, filed on November 10, 1998. U.S. Patent Nos. 5,711,784 and 5,944,872, and U.S. Provisional Application Nos. 60/107,797 and 60/109,443 are herein incorporated by reference.

### *Field of the Invention*

This invention pertains to methods for recovering metals, such as nickel and cobalt, from metal-rich soil using phytoextracting or phytomining techniques. Metals can be selectively extracted from soil by cultivating certain metal hyperaccumulating plants, such as *Alyssum* plants, on soil treated to adjust the pH.

***Related Art***

Industrial practices such as mining, smelting and disposing of manufacturing wastes have increased the concentrations of toxic metals in the environment. For example, at many nickel mining and smelting sites, levels of nickel and cobalt in soil have become so high that few plants survive, resulting in severe disruption of local ecosystems. Once nickel and cobalt enter soil, their removal is difficult since they are relatively immobile and they do not degrade into less toxic substances. The size of the areas affected by smelter and mine wastes are usually so large that engineering methods of soil remediation, such as soil removal and replacement, are too expensive to be practical (Cunningham *et al.*, "Phytoremediation of Contaminated Soils," *Trends Biotechnol.* 13: 393-397 (1995)).

The ability of certain plants to grow in metal-containing or metal-contaminated soil, and to actively accumulate heavy metals in their tissues, has created an interest in using such plants to extract metals from soil. Growing plants, including crops, on contaminated soil to extract contaminants is referred to as phytoextraction. This method is particularly effective in arable contaminated soils because it causes little disruption or dispersal, while preserving soil fertility and landscapes.

Nickel is one of the most widely found, and technologically important metals. It is a natural constituent in all soils, being particularly high in concentration in certain types of soil and geological materials such as serpentine, lateritic serpentine, ultramafic and meteor-derived soils. Cobalt, another valuable metal, has chemical and geological characteristics very similar to nickel and is generally found in the same soils. Other metals that may be found in such soils include those of the platinum and palladium families such as palladium, rhodium, ruthenium, platinum, iridium, osmium and rhenium, and metals such as selenium, zinc and cadmium.

Sites containing serpentine, lateritic serpentine, ultramafic and meteor-derived soils and materials can be conventionally mined or cultivated with metal-accumulating plants. Using such plants to extract metals from mineralized (geogenic) soils is referred to as phytomining.

5 U.S. Patent No. 5,364,451 to Raskin *et al.*, is directed to a method of remediating polluted soils at a reduced cost. Raskin *et al.* remove metals from metal-rich soil by growing plants of the family Brassicaceae in the metal-rich soil. While Raskin *et al.* generally describe a variety of plants and a large number of metals that may be recovered, the examples mainly describe the recovery of  
10 chromium and lead from genetically altered plants. Thus, although promising, Raskin *et al.* offer little basis for an opportunity to proceed directly with soil phytomining or phytoextraction through plant growth or cultivation.

U.S. Patent No. 5,785,735 to Raskin *et al.*, is also directed to methods of remediating polluted soils. Raskin *et al.* remove metals from metal-rich soil by  
15 growing crop and crop-related members of the plant family Brassicaceae in the metal-rich soil. The methods require the formation of a complex between the metal and a chelating agent added to the soil, the application of an electric field to the soil or a reduction in the pH of the soil. While Raskin *et al.* generally describe a variety of plants, the specification mainly describes the recovery of  
20 metals from genetically altered plants. Thus, again, Raskin *et al.* offer little basis for an opportunity to proceed directly with soil phytomining or phytoextraction through plant growth or cultivation.

Scientists recognize that increasing the pH of soil decreases the ability of farm crops to take-up heavy metals. U.S. Patent No. 5,711,784 to Chaney *et al.*  
25 reflects the belief in the art that reducing the pH of the soil "increases the phytoavailability of nickel and cobalt." As disclosed by Chaney *et al.*, a "reduced pH increases solubility, and optimizes the release of these metals for absorption by the roots and translocation to the above-ground tissues of the plant." However, reducing the pH of the soil also renders the metals more mobile and may allow  
30 for further contamination of the site. Therefore, cultivating plants which are

hyperaccumulators of nickel, cobalt and other metals through phytoextraction or phytomining, is a desirable alternative as a means for recovering such metals.

### ***Summary of the Invention***

Accordingly, this invention relates to improved systems for recovering metals by phytomining or phytoextracting soils rich in metals.

The invention further relates to increasing nickel uptake by plants used in phytomining and phytoextraction by elevating the soil pH. Nickel is ultimately recovered from plant tissues at economically acceptable levels without further contaminating the nickel-containing site.

The invention further relates to lowering the pH in soils prior or subsequent to nickel recovery to collect, for example, cobalt or any other metal present in the metal-laden soil.

In a particular aspect of the invention, *Alyssum* plants are cultivated under favorable pH conditions to selectively accumulate certain metals relative to other metals.

The invention further relates to a method for selectively increasing the amount of at least one metal recovered from metal-containing soil comprising:

- (a) elevating or lowering the pH of the soil;
- (b) cultivating at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one metal from the soil in above-ground tissue;
- (c) elevating the pH of the soil if the pH was lowered in step (a) or lowering the pH of the soil if the pH was elevated in step (a); and
- (d) cultivating the at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one second metal from the soil in above-ground tissue.

The invention further relates to a method for recovering nickel from nickel-containing soil comprising:

- (a) elevating the pH of the soil;
- (b) cultivating at least one nickel-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel;
- 5 (c) harvesting said at least one plant; and
- (d) recovering nickel from said harvested plant.

The invention further relates to a method for recovering cobalt from cobalt-containing soil comprising:

- (a) lowering the pH of the soil;
- 10 (b) cultivating at least one cobalt-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is cobalt;
- (c) harvesting said at least one plant; and
- (d) recovering cobalt from said harvested plant.

15 The invention further relates to the identification of new hyperaccumulating species of *Alyssum* whereby collected plants are screened by comparing nickel-uptake by the plants to nickel-uptake by the bench-mark nickel-hyperaccumulator *A. murale* 103. These new metal-hyperaccumulating species, cultivated on nickel-containing soil, accumulate nickel in above-ground tissue at a concentration of 1.55% or greater by weight based on the gross dry weight of the tissue.

The invention further relates to seeds of the *Alyssum* plant species.

The invention further relates to pollen of the *Alyssum* plant species.

25 The invention further relates to plants that have all the physiological and morphological characteristics of the *Alyssum* plant species.

The invention further relates to propagation material of the *Alyssum* plant species.

30 The invention further relates to a method for decontaminating metal-containing soil, comprising cultivating at least one hyperaccumulator plant in metal-containing soil, whereby the concentration of metal in the above-ground



plant tissue of the at least one hyperaccumulator plant exceeds the concentration of metal in the soil by a factor of at least 2.

### ***Detailed Description of the Preferred Embodiments***

5 In the present invention, it was discovered that certain metals can be selectively recovered from metal-rich soil using phytoextraction or phytomining techniques employing plants classified as hyperaccumulators of metals. By cultivating selected plants on metal-containing soil, the metals absorbed by the roots can be translocated to above-ground tissues, such as the stems, leaves, flowers and other leaf and stem tissues. This feature facilitates recovery of the metal extracted from the soil. Metal concentrations can be as high as about 5.0% in above-ground plant tissues, when leaves are included, which renders the metal recovery very economical. However, recovering metal in concentrations of less than about 5.0%, such as about 4.0%, 3.0%, 2.5%, 1.0% or 0.1% remains useful. For example, a recovery of about 1.0% or more offers economic return for decontaminating polluted soil and for phytomining. A recovery of about 0.1% to about 1.0% of cobalt is sufficient to decontaminate polluted soil at a low cost, and a recovery of even less than about 0.1% of some metals can still effectively decontaminate polluted soils.

20 The invention further relates to a method for selectively increasing the amount of at least one metal recovered from metal-containing soil comprising:

- (a) elevating or lowering the pH of the soil;
- (b) cultivating at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one metal from the soil in above-ground tissue;
- 25 (c) elevating the pH of the soil if the pH was lowered in step (a) or lowering the pH of the soil if the pH was elevated in step (a); and

(d) cultivating the at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one second metal from the soil in above-ground tissue.

The invention further relates to a method for recovering nickel from nickel-containing soil comprising:

- (a) elevating the pH of the soil;
- (b) cultivating at least one nickel-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel;
- (c) harvesting said at least one plant; and
- (d) recovering nickel from said harvested plant.

The invention further relates to a method for recovering cobalt from cobalt-containing soil comprising:

- (a) lowering the pH of the soil;
- (b) cultivating at least one cobalt-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is cobalt;
- (c) harvesting said at least one plant; and
- (d) recovering cobalt from said harvested plant.

The invention further relates to the identification of new hyperaccumulating species of *Alyssum* whereby collected plants are screened by comparing nickel-uptake by the plants to nickel-uptake by the bench-mark nickel-hyperaccumulator *A. murale* 103. These new metal-hyperaccumulating species, cultivated on nickel-containing soil, accumulate nickel in above-ground tissue at a concentration of 1.55% or greater by weight based on the gross dry weight of the tissue.

The invention further relates to seeds of the *Alyssum* plant species.

The invention further relates to pollen of the *Alyssum* plant species.

The invention further relates to plants that have all the physiological and morphological characteristics of the *Alyssum* plant species.

The invention further relates to propagation material of the *Alyssum* plant species.

The invention further relates to a method for decontaminating metal-containing soil, comprising cultivating at least one hyperaccumulator plant in metal-containing soil, whereby the concentration of metal in the above-ground plant tissue of the at least one hyperaccumulator plant exceeds the concentration of metal in the soil by a factor of at least 2, preferably by a factor of 2, 3 or 4.

In a preferred aspect of the invention, nickel is selectively accumulated by growing one or more nickel-hyperaccumulating plants in metal-rich, e.g., nickel-rich, soil and elevating the pH of the soil. The pH of the soil may be elevated before, during or after the plants are cultivated. Preferably, the pH is elevated prior to plant cultivation. Thus, the invention relates to the surprising discovery that raising the pH of the metal-rich soil favors nickel accumulation in plant tissue over other metals. The soil pH can then be lowered to selectively accumulate, in the plant tissue, other metals such as cobalt. The preferred pH will depend, *inter alia*, upon the particular metal and the soil. For example, the preferred pH for nickel extraction ranges between about 6.3 and about 7.0 when the soil is a serpentine soil or when the soil contains high iron oxide levels. The most preferred pH ranges from about 6.3 to about 6.7. However, when the iron oxide level is low, a more alkaline pH may be used.

Cobalt extraction is also affected by the soil chemistry. For example, the most preferred pH for cobalt extraction is about 5.5 when aluminum and/or manganese are present in the soil. For metal extraction in general, the preferred pH ranges between about 5.5 and about 7.0.

Soil pH can be raised and lowered with bases and acids. Such bases and acids may be either naturally occurring or synthetic. To raise the pH, bases such as limestone (calcitic ( $\text{CaCO}_3$ ) or dolomitic ( $\text{CaMgCO}_3$ )), lime ( $\text{CaO}$ ), hydrated lime ( $\text{Ca(OH)}_2$ ), industrial, municipal or agricultural alkaline by-products that contain any of the above bases or a limestone equivalent, or the like can be used.

The phrase "limestone equivalent" is intended to encompass bases that have the

same alkalinity as limestone. To lower the pH, acids such as organic and inorganic acids can be used. Examples of such organic and inorganic acids include acetic acid, aqueous hydrogen chloride, aqueous sulfuric acid, sulfur, ammonium, urea-containing fertilizers, nitric acid, sulfide minerals, including, but not limited to, pyrite, and the like.

The amount of base or acid to add depends upon the existing pH of the soil and the soil chemistry. Methods used to determine the amount include, but are not limited to, adding acid or a base, such as  $\text{CaCO}_3$ , to the soil sample and measuring the resulting pH, then drawing a pH response curve to extrapolate the amount needed to obtain the desired pH.

After cultivation, the hyperaccumulator plant is harvested in a conventional fashion, i.e., by cutting the plant at soil level. The harvested materials are then left to dry in the field in the manner in which hay is dried. Alternatively, the harvested materials are dried in much the same fashion that alfalfa is dried, so as to remove most of the water present in the plant tissue by forced heated air drying. After drying, the plant tissue is collected by normal agricultural practices of hay-making, incinerated and reduced to an ash with or without energy recovery. Alternatively, the dried plant material may be hydrolyzed with concentrated acid to produce sugars and the metals recovered according to U.S. Patent Nos. 5,407,817, 5,571,703 and 5,779,164. The sugars may then be fermented to produce ethanol.

The resulting dried plant material may alternatively be further treated by known roasting, sintering or smelting methods which allow the metals in the ash or ore to be recovered according to conventional metal refining methods such as acid dissolution and electrowinning.

Conventional smelting, roasting and sintering temperatures from about 260°C to about 1000°C are sufficient to combust the dried plant material to oxidize and vaporize the organic material present and to prevent dioxin accumulation during incineration. The preferred temperature is sufficient to remove the organic carbon to free the ash. The most preferred temperature is

about 1000°C. The process leaves a residue of the accumulated metal with few contaminants known to interfere with metal refining. Further, it is expected that the concentration of other components in the ash will be much lower than with conventional mined ore concentrates. For example, serpentine laterite ores generally contain over 10,000 ppm (1%) Fe whereas a biomass obtained using phytomining techniques only contains about 100-500 ppm (0.01 - 0.05%) Fe.

By definition, nickel-hyperaccumulating plants accumulate at least about 1000 mg of nickel per 1 kg dry weight of plant tissue (obtained from a plant grown in soil where the plant naturally occurs). Similarly, cobalt-hyperaccumulating plants are defined as plants that accumulate at least about 1000 mg of cobalt per 1 kg dry weight of plant tissue (obtained from a plant grown in soil where the plant naturally occurs). However, zinc- and manganese-hyperaccumulators are defined as plants that accumulate at least about 10,000 mg of zinc and manganese, respectively, per 1 kg dry weight of plant tissue (obtained from a plant grown in soil where the plant naturally occurs). Finally, cadmium-hyperaccumulators are defined as plants that accumulate at least about 100 mg cadmium per 1 kg dry weight of plant tissue (obtained from a plant grown in soil where the plant naturally occurs).

By screening a wide variety of plants, those of the *Alyssum* genus (Brassicaceae family) have been identified as hyperaccumulators of nickel. These plants also naturally accumulate cobalt and may accumulate metals such as Zn, Mn and Cd, and metals from the platinum and palladium families including Pd, Rh, Ru, Pt, Ir, Os and Re.

More specifically, plants which naturally concentrate nickel in above-ground tissues and generally exhibit an enhanced uptake of cobalt and other metals include members of the section *Odontarrhena* of the genus *Alyssum*. The metals accumulate in nickel-hyperaccumulating *Alyssum* plant species when the plants are grown in contaminated soils. Some 48 taxa within the section *Odontarrhena* of the genus *Alyssum* are known to be hyperaccumulators of nickel. These include the following species: *A. akamasicum*, *A. alpestre*, *A. anatolicum*,

5 *A. callichroum*, *A. cassium*, *A. chondrogynum*, *A. cilicicum*, *A. condensatum*, *A.*  
*constellatum*, *A. crenulatum*, *A. cypricum*, *A. davisianum*, *A. discolor*, *A.*  
*dubertretii*, *A. eriophyllum*, *A. euboicum*, *A. floribundum*, *A. giosnanum*, *A. huber-*  
*morathii*, *A. janchenii*, *A. markgrafii*, *A. masmenaeum*, *A. obovatum*, *A.*  
*oxycarpum*, *A. penjwinensis*, *A. pinifolium*, *A. pterocarpum*, *A. robertianum*, *A.*  
*samariferum*, *A. singarense*, *A. smolikanum*, *A. syriacum*, *A. trapeziforme*, *A.*  
*troodii*, *A. virgatum*, *A. murale*, *A. pintodasilvae* (also known as *A. serpyllifolium*  
*var. lusitanicum*), *A. serpyllifolium*, *A. malacitanum* (also known as *A.*  
*serpyllifolium var. malacitanum*), *A. lesbiacum*, *A. fallacinum*, *A. argenteum*, *A.*  
10 *bertolonii*, *A. tenium*, *A. heldreichii*, *A. corsicum*, *A. pterocarpum* and *A. caricum*  
as well as newly discovered species such as *A. corsicum* G16, *A. murale* G69 and  
*A. murale* G82. These species were deposited on November 6, 1998, under the  
provisions of the Budapest Treaty at the American Type Culture Collection,  
10801 University Blvd., Manassas, VA 20110-2209, and assigned ATCC nos.  
15 203436, 203437 and 203438, respectively.

Species of *Alyssum* that naturally accumulate nickel in amounts of up to  
20% greater than any known *Alyssum* hyperaccumulator have been isolated.  
Species *A. murale* G49, *A. murale* G54, *A. murale* G69 and *A. murale* G82  
isolated in Greece and species *A. corsicum* G16 isolated in Turkey all accumulate  
20 nickel in amounts greater than the known species *A. murale* 103 which  
accumulates nickel such that nickel makes up 1.14% by dry weight of a plant  
shoot from a test field of serpentine soil. The new hyperaccumulators accumulate  
nickel in amounts such that 1.55-1.60% by dry weight of the shoot is nickel. The  
results of nickel accumulation of these five new accumulators relative to the  
25 benchmark accumulator *A. murale* 103 is shown in Example 4.

About 250 other plant taxa, including those of tropical origin, have been  
shown to accumulate quantities of nickel and other metals. However, many of  
these plants do not exceed about 10,000 mg of metal per kg of plant tissue dry  
weight. Other metal-accumulating plants includes species of the genus *Cyanotis*  
30 such as *Cyanotis longifolia*; species of the genus *Bulbostylis* such as *Bulbostylis*

*mucronata*; species of the genus *Combretum* such as *Combretum decandrum*; species of the genus *Crassula* such as *C. alba*, *C. vaginata* and *C. argyrophylla*; species of the genus *Clethra* such as *Clethra barbinervis*; plants from the Cunoniaceae family such as species of the genus *Geissois* including *G. intermedia*, *G. magnifica*, *G. montana*, *G. pruinosa*, *G. trifoliata* and *G. racemosa*; species of the genus *Argophyllum*; members of Brassicaceae family such as species of the genus *Thlaspi* such as *Thlaspi caerulescens*, *Thlaspi montanum* var. *montanum* and *Thlaspi montanum* var. *siskiyouense*; species of the genus *Serpentine* such as *Serpentine polygaloides*; species of the genus *Sebertia* such as *Sebertia acuminata*; species of the genus *Hybanthus* such as *Hybanthus floribundas*; species of the genus *Psychotria* such as *Psychotria douarrei*; species of the genus *Rinorea* such as *Rinorea bengalensis*; species of the genus *Pearsonia* such as *Pearsonia metallifera*; species of the genus *Sebertia* such as *Sebertia acuminata*; and species of the following genera: *Homalium*, *Myristica*, *Trichospermum*, *Planchonella* and *Peltaria*. Additional plants include, but are not limited to, *Streptanthus polygaloides*, *Berkheya coddii*, *Phyllanthus palawanensis*, *Dichapetalum gelonioides* ssp. *tuberculatum* and *Stackhousia tryonii*.

Additional metal hyperaccumulators are listed below:

#### ACANTHACEAE

*Blepharis acuminata*, *Justicia lanstykii*, *Lophostachys villosa*, *Phidiasia lindavii*, *Ruellia geminiflora*

#### ADIANTACEAE

*Adiantum* sp.

#### ANACARDIACEAE

*Rhus wildii*

#### ASTERACEAE

*Berkheya coddii*, *Chromolaena* sp. cf. *meyeri*, *Dicoma niccolifera*, *Gochnatia crassifolia*, *G. recurva*, *Koanophyllon grandiceps*, *K. prinodes*, *Leucanthemopsis alpina*, *Pentacalia*, *Senecio* *Senecio pauperculus*, *Shafera platyphylla*, *Solidago hispida*

#### BORAGINACEAE

*Heliotropium* sp.

BRASSICACEAE

*Bornmuellera*, *Cardamine resedifolia*, *Cochlearia aucheri*, *C. sempervivum*,  
*Peltaria emarginata*, *Streptanthus polygaloides*

5

BUXACEAE

*Buxus*

CAMPANULACEAE

*Campanula scheuchzeri*, *Arenaria*, *Minuartia laricifolia*, *M. verna*

10

CLUSIACEAE

*Garcinia bakeriana*, *G. polyneura*, *G. revoluta*, *G. ruscifolia*

CONVOLVULACEAE

*Merremia xanthophylla*

CUNONIACEAE

*Pancheria engleriana*

15

DICHAPETALACEAE

*Dichapetalum gelonioides* and ssp. *tuberculatum* and ssp. *andamanicum*

DIPTEROCARPACEAE

*Shorea tenuiramulosa*

20

ESCALLONIACEAE

*Argophyllum grunowii*, *A. laxum*

EUPHORBIACEAE

*Baloghia* sp., *Bonania*, *Cleidion viellardii*, *Cnidoscolus* sp. cf. *bahianus*,  
*Euphorbia*, *Gymnanthes recurva*, *Leucocroton*, *Phyllanthus*, *Sapium*  
*erythrospermum*, *Savia*

25

FABACEAE

*Anthyllis* sp., *Pearsonia metallifera*, *Trifolium pallescens*

FLACOURTIACEAE

*Casearia silvana*, *Homalium*, *Xylosma*

30

JUNCACEAE

*Luzula lutea*

MELIACEAE



*Walsura monophylla*

MYRISTICACEAE

*Myristica laurifolia*

MYRTACEAE

- 5 *Mosiera araneosa*, *M. ekmanii*, *M. x miraflorensis*, *M. ophitica*, *Psidium araneosum*, *P. havanense*

OCHNACEAE

*Brackenridgea palustris* and ssp. *foxworthyi* and ssp. *kjellbergii*, *Ouratea nitida*, *O. striata*

- 10 OLEACEAE

*Chionanthus domingensis*

ONCOTHECACEAE

*Oncotheca balansae*

POACEAE

- 15 *Trisetum distichophyllum*

RANUNCULACEAE

*Ranunculus glacialis*

RUBIACEAE

- 20 *Ariadne shaferi* ssp. *shaferi* and ssp. *moaensis*, *Mitracarpus* sp., *Phyllomelia coronata*, *Psychotria clementis*, *P. costivenia*, *P. douarrei*, *P. glomerata*, *P. osseana*, *P. vanhermanii*, *Rondeletia*

SAPOTACEAE

*Planchonella oxyedra*, *Sebertia acuminata*

SAXIFRAGACEAE

- 25 *Saxifraga*

SCROPHULARIACEAE

*Esterhazyia* sp. and *Linaria alpina*

STACKHOUSIACEAE

*Stackhousia tryonii*

- 30 TILIACEAE

*Tetralix brachypetalus*, *T. cristalensis*, *T. jaucoensis*, *T. moaensis*, *T. nipensis*, *Trichospermum kjellbergii*

TURNERACEAE

*Turnera subnuda*

VELLOZIACEAE

*Vellozia* sp.

5

VIOLACEAE

*Agatea deplanchei*, *Hybanthus*, *Rinorea bengalensis*, *R. javanica*, *Rinorea* sp.

ACERACEAE

*Acer pseudoplatanus*

10

BRASSICACEAE

*Cardaminopsis halleri*, *Thlaspi avalanum*, *T. brachypetalum*, *T. caerulescens*, *T. ochroleucum*, *T. rotundifolium* subsp. *cepaefolium*, *T. praecox*, *T. stenopterum*, *T. tatrense*

CARYOPHYLLACEAE

15

*Minuartia verna*, *Polycarpaea synandra*

CISTACEAE

*Cistus incanus* ssp. *creticus*

DICHAPETALACEAE

*Dichapetalum gelonioides*

20

PLUMBAGINACEAE

*Armeria maritima* var. *halleri*

POACEAE

*Agrostis stolonifera*, *A. tenuis*, *Arrhenatherum elatius*, *Festuca ovina*

POLYGONACEAE

25

*Rumex acetosa*

VIOLACEAE

*Viola calaminaria*

AMARANTHACEAE

*Pandiaka metallorum*, *Celosia trigyna*

30

ASTERACEAE

*Anisopappus chinensis*, *A. davyi*, *Gutenbergia pubescens*, *Millotia myosotidifolia*, *Vernonia petersii*

CARYOPHYLLACEAE

*Minuartia verna* ssp. *hercynica* and *Silene cobalticola*

COMMELINACEAE

*Commelina zigzag* and *Cyanotis longifolia*

5 CONVOLVULACEAE

*Ipomoea alpina*

CRASSULACEAE

*Crassula alba* and *C. vaginata*

10 CYPERACEAE

*Ascolepis metallorum*, *Bulbostylis cupricola*, *B. pseudoperennis*

EUPHORBIACEAE

*Monadenium cupricola* and *Phyllanthus williamoides*

FABACEAE

*Crotalaria cobalticola* and *Vigna dolomitica*

15 IRIDACEAE

*Gladiolus gregarius*

LAMIACEAE

*Aeollanthus subacaulis* var. *linearis*, *A. homblei*, *A. saxatilis*, *A. subacaulis* var. *ericoides* and var. *linearis*, *Becium grandiflorum* var. *vanderystii*, *Haumaniastrum homblei*, *H. katangense*, *H. robertii*, *H. rosulatum*

20

MALVACEAE

*Hibiscus rhodanthus*

PINACEAE

25

*Abies balsamea*

POACEAE

*Eragrostis racemosa*, *Rendlia altera*, *Sporobolus congoensis*

PTERIDACEAE

*Actiniopteris* sp.

30 SCROPHULARIACEAE

*Alectra sessiliflora* var. *senegalensis*, *Buchnera henriquesii*, *Crepidorhopalon tenuisa*, *C. perennisa*, *Sopubia mannii*, *S. metallorum*, *S. neptunii*, *Striga hermontheca*

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TILIACEAE

*Triumfetta dekindtiana*, *T. digitata*, *T. welwitschii* var. *descampii*

VELLOZIACEAE

*Xerophyta retinervis* var. *equisetoides*

5

APOCYNACEAE

*Alyxia rubricaulis*

CELASTRACEAE

*Maytenus bureaviana*, *M. pancheriana*, *M. sebertiana*

10

CLUSIACEAE

*Garcinia amplexicaulis*

MYRTACEAE

*Eugenia clusioides*

PROTEACEAE

*Beaupreopsis paniculata*, *Macadamia angustifolia*, *M. neurophylla*

15

ASTERACEAE

*Haplopappus fremontii*, *Machaeranthera glabriuscula*, *M. ramosa*, *M. venusta*

BRASSICACEAE

*Stanleya pinnata*, *S. bipinnata*

20

CHENOPODIACEAE

*Atriplex confertifolia*

LECYTHIDACEAE

*Lecythis ollaria*

LEGUMINOSAE

25

*Acacia cana*, *Astragalus bisulcatus*, *A. osterhoutii*, *A. pattersonii*, *A. pectinatus*,  
*A. racemosus*, *Neptunia amplexicaulis*

RUBIACEAE

*Morinda reticulata*

30

SCROPHULARIACEAE

*Castilleja chromosa*

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The metals accumulated include nickel, cobalt, barium, gold, beryllium, mercury, molybdenum, copper, arsenic, selenium, antimony, manganese, silver, thallium, tin, lead, rubidium, chromium, cerium, vanadium, cesium, uranium, plutonium, strontium, yttrium, technetium, iridium, ruthenium, palladium, rhodium, platinum, osmium, rhenium, zinc and cadmium.

Metal sequestration can be improved by optimizing soil calcium concentration, using ammonium-containing or ammonium-generating fertilizers rather than other nitrate-containing fertilizers, and by applying chelating agents to the soil in which the hyperaccumulator plants are grown.

*Alyssum* species which hyperaccumulate metals such as nickel and cobalt evolved in nickel-rich ultramafic and serpentine soils which have low soil calcium and a low Ca:Mg ratio. It is now known that the presence of extremely low and extremely high calcium concentrations in soil inhibits nickel hyperaccumulation by *Alyssum*. See PCT/US97/15109. Acceptable calcium concentrations in soil range from about 0.128 mM to about 5.0 mM. In terms of percentages, an acceptable calcium concentration in soil ranges from about 2% to about 80% of the exchangeable cations. A preferable range is from about 10% to about 80% of the exchangeable cations. The most preferred range is from about 30% to about 70% of the exchangeable cations. Such ranges can be achieved, if necessary, by adding calcium-containing agents to the soil such as limestone. In addition, gypsum could be added to the soil to raise the exchangeable calcium of the soil to benefit nickel accumulation.

The presence of intermediate concentrations of calcium, i.e., between about 0.128 mM and about 5.0 mM, increases nickel uptake whereas calcium values of about 0.128 mM and below, or about 5 mM and above, decrease nickel uptake. Combined with an exchangeable Ca:Mg ratio of between about 0.16 and about 0.40, much lower than recommended, an additional increase in nickel concentration in plant tissues is observed. By "exchangeable Ca:Mg ratio" is intended the ratio of extractable calcium and magnesium in the soil.

Although hyperaccumulators such as *Alyssum* have developed the ability to hyperaccumulate metals in above-ground tissues, fertilizer supportive of growth, particularly in polluted soil, can be used as an additive to increase hyperaccumulation. Ammonium fertilizers localize acidification adjacent to the root which aids hyperaccumulation of various metals such as Ni, Zn, Cd, Co, etc. The use of ammonium fertilizers *per se* is well-known, and acceptable fertilizers and protocols can be readily determined with no more than routine experimentation, by those of ordinary skill in the art. Other additives include, but are not limited to, nutrients such as phosphate which helps to maximize the yield of nickel, for example.

Another possible additive to the contaminated soil is a metal chelating agent. Metal chelates are commonly used in agriculture and occur naturally in living cells. The addition of chelating agents, such as nitrolotriactic acid (NTA), ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(p-aminoethylether-N, N-tetraacetic acid) or any of a variety of amino-acetic acids known to those of ordinary skill in the art as chelating agents, to the soil to be phytomined or phytoextracted improves the movement of soil metals to root surfaces for uptake and translocation into above-ground tissues. Preferred chelating agents are NTA or EDTA. Typically, chelating agents will be added at a concentration ranging from about 0.5 to about 10 millimoles per kg soil. As with the use of fertilizers, the optimum concentration of chelating agents can be readily determined with no more than routine experimentation. Chelating compounds which chelate nickel in the presence of high soil levels of Fe, Mg and Ca selectively increase nickel uptake by hyperaccumulator plants.

The following examples are illustrative, but not limiting, of the methods of the present invention. Other suitable modifications and adaptations of the variety of conditions normally encountered which are obvious to those skilled in the art are within the spirit and scope of the present invention.

## *Examples*

### *Example 1*

*A. murale* 103 plants were grown in sets of two for 120 days in 19 pot-sets (4 L) of contaminated or serpentine soils (Mg-nitrate was leached out) without acidification, the first pot in a set, and with acidification, the second pot in a set. Water was maintained near field capacity by daily watering with deionized water. The plants were cultivated at a temperature of about 28°C during the day and about 20°C at night. The soils were acidified using nitric acid and the pH was raised using powdered reagent-grade CaCO<sub>3</sub>. The soils included serpentine soils rich in nickel (containing from about 100 to about 5000 ppm nickel) obtained from southwest Oregon (soils 3-19), nickel-refinery contaminated Welland loam from Port Colborne, Ontario (soil 1) and nickel-refinery contaminated Quarry muck from Port Colborne, Ontario (soil 2). Fertilizers containing, *inter alia*, nickel, potassium, sulfur and phosphorous, were added to optimize plant growth.

Table 1 shows the results of the experiment in contaminated soil.

*Table 1*

| Soil | TRT | Final<br>pH | Yield                         | Ni    | Co   | Mn    | Zn   | Cu   | Fe  |
|------|-----|-------------|-------------------------------|-------|------|-------|------|------|-----|
|      |     |             | g shoot<br>dry<br>matter/ pot | mg/kg |      |       |      |      |     |
| 1    | 2   | 5.16        | 27.4                          | 9150  | 119  | 82.4  | 117  | 15.0 | 58  |
| 1    | 6   | 4.96        | 22.7                          | 4220  | 84.7 | 145.6 | 180  | 19.5 | 64  |
| 2    | 2   | 6.04        | 40.9                          | 4570  | 5.9  | 20.9  | 99.0 | 4.0  | 68  |
| 2    | 6   | 5.40        | 28.8                          | 2150  | 7.1  | 63.0  | 142  | 6.5  | 82  |
| 3    | 2   | 6.26        | 21.5                          | 6370  | 19.9 | 68.8  | 61.5 | 3.5  | 160 |
| 3    | 6   | 5.38        | 19.7                          | 6480  | 308  | 680   | 65.9 | 5.5  | 260 |
| 4    | 2   | 5.61        | 19.6                          | 12400 | 56.5 | 181   | 88.0 | 4.0  | 332 |
| 4    | 6   | 5.21        | 15.6                          | 8560  | 377  | 140   | 135  | 5.0  | 345 |
| 5    | 2   | 5.88        | 24.0                          | 1860  | 6.0  | 53.0  | 252  | 3.2  | 137 |
| 5    | 6   | 5.32        | 21.1                          | 1220  | 9.8  | 153   | 379  | 3.5  | 121 |
| 6    | 2   | 6.03        | 24.5                          | 4580  | 14.6 | 84.2  | 61.2 | 5.2  | 183 |
| 6    | 6   | 5.42        | 27.2                          | 5040  | 58.5 | 227   | 70.3 | 5.5  | 195 |
| 7    | 2   | 5.54        | 23.3                          | 5750  | 36.3 | 134   | 83.7 | 5.0  | 250 |
| 7    | 6   | 5.28        | 23.2                          | 4870  | 86.8 | 272   | 77.9 | 5.5  | 274 |
| 8    | 2   | 5.77        | 21.1                          | 9630  | 28.8 | 130   | 52.6 | 4.0  | 223 |
| 8    | 6   | 5.21        | 17.5                          | 7180  | 94.0 | 291   | 74.9 | 4.8  | 221 |
| 9    | 2   | 6.12        | 22.1                          | 9770  | 38.7 | 122   | 69.6 | 4.8  | 240 |
| 9    | 6   | 5.62        | 22.5                          | 9100  | 196  | 532   | 69.7 | 5.2  | 273 |
| 10   | 2   | 6.25        | 20.0                          | 12900 | 31.2 | 109   | 79.3 | 2.5  | 318 |
| 10   | 6   | 5.76        | 19.3                          | 11500 | 182  | 774   | 93.5 | 3.2  | 412 |
| 11   | 2   | 5.72        | 32.8                          | 8460  | 37.3 | 148   | 75.5 | 5.0  | 266 |
| 11   | 6   | 5.35        | 24.3                          | 6010  | 136  | 339   | 93.6 | 4.8  | 230 |
| 12   | 2   | 6.54        | 20.3                          | 8070  | 29.0 | 84.4  | 74.0 | 3.5  | 222 |
| 12   | 6   | 5.78        | 18.4                          | 8240  | 86.0 | 186   | 66.5 | 3.2  | 178 |
| 13   | 2   | 6.34        | 18.8                          | 11000 | 16.2 | 39.1  | 51.8 | 2.2  | 186 |
| 13   | 6   | 5.87        | 19.6                          | 9970  | 36.0 | 103   | 56.6 | 2.8  | 181 |
| 14   | 2   | 5.68        | 21.3                          | 9150  | 67.0 | 331   | 65.8 | 4.8  | 278 |

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| Soil | TRT | Final pH | Yield                   | Ni    | Co   | Mn  | Zn   | Cu  | Fe  |
|------|-----|----------|-------------------------|-------|------|-----|------|-----|-----|
|      |     |          | g shoot dry matter/ pot | mg/kg |      |     |      |     |     |
| 14   | 6   | 4.84     | 13.3                    | 5820  | 313  | 957 | 86.0 | 4.8 | 567 |
| 15   | 2   | 6.04     | 19.4                    | 7620  | 30.5 | 142 | 69.8 | 4.8 | 365 |
| 15   | 6   | 5.94     | 23.7                    | 6110  | 463  | 820 | 88.6 | 4.8 | 220 |
| 16   | 2   | 6.07     | 21.0                    | 3090  | 47.4 | 128 | 89.1 | 6.8 | 172 |
| 16   | 6   | 5.41     | 18.2                    | 3560  | 225  | 563 | 105  | 8.0 | 267 |
| 17   | 2   | 6.02     | 20.6                    | 9080  | 37.5 | 124 | 114  | 3.8 | 256 |
| 17   | 6   | 5.63     | 23.9                    | 7940  | 262  | 973 | 127  | 4.2 | 252 |
| 18   | 2   | 5.99     | 19.4                    | 11600 | 35.3 | 127 | 68.5 | 3.0 | 440 |
| 18   | 6   | 5.53     | 15.4                    | 9500  | 204  | 908 | 116  | 4.2 | 548 |
| 19   | 2   | 5.59     | 21.8                    | 436   | 19.1 | 259 | 92.4 | 7.8 | 190 |
| 19   | 6   | 5.11     | 19.5                    | 584   | 72.4 | 929 | 112  | 8.8 | 156 |

"TRT" = treatment. In treatment 2, the soil pH was not adjusted. In treatment 6, the soil pH was acidified.

As illustrated in Table 1, the plants grown on soils of less acidic pH generally accumulated far greater amounts of nickel than the plants grown on more acidic soils. In addition, plants taking up larger amounts of nickel on less acidic soils accumulated smaller amounts of other metals such as cobalt, manganese and zinc which are commonly found in lower concentrations in shoots after soil pH is raised.

### Example 2

To validate the above example and to obtain optimization, *Alyssum* plants were grown on nickel-refinery contaminated Welland loam (soil 1), wherein the pH was elevated by applying limestone (Table 2). The plants were also grown on nickel-refinery contaminated Quarry muck (soil 2) and serpentine soils (soils 3-

11) (Table 3). The same cultivation conditions recited in Example 1 were used in Example 2.

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**Table 2**

Effect of phosphate, pH and Ca:Mg variation on geometric mean shoot yield and micronutrient composition of two *Alyssum* species grown on nickel-refinery contaminated Welland loam (soil 1) for 120 days.

| Soil                     | TRT | Yield   | Ni      | Co      | Mn      | Zn      |
|--------------------------|-----|---------|---------|---------|---------|---------|
|                          |     | g/pot   | g/kg    | mg/kg   | mg/kg   | mg/kg   |
| 1                        | 1   | 6.68 b* | 7.61 a  | 127 a   | 23.7 e  | 157 fg  |
| <u>Phosphate Series:</u> |     |         |         |         |         |         |
| 1                        | 3   | 7.82 ab | 5.94 bc | 118 ab  | 72.8 c  | 209 ab  |
| 1                        | 2   | 9.78 ab | 5.49 cd | 109 bcd | 59.3 d  | 170 def |
| 1                        | 4   | 8.71 ab | 6.40 b  | 114 a-d | 66.7 cd | 178 c-f |
| 1                        | 5   | 8.03 ab | 5.97 bc | 98.8 d  | 60.8 cd | 169 def |
| <u>pH Series:</u>        |     |         |         |         |         |         |
| 1                        | 6   | 8.14 ab | 3.93 e  | 132 a   | 177 a   | 217 a   |
| 1                        | 7   | 7.46 ab | 4.93 d  | 119 ab  | 99.8 b  | 183 b-e |
| 1                        | 2   | 9.78 ab | 5.49 cd | 109 bcd | 59.3 d  | 170 deg |
| 1                        | 8   | 10.4 a  | 8.47 a  | 101 cd  | 19.1 f  | 142 g   |
| <u>Ca:Mg Series:</u>     |     |         |         |         |         |         |
| 1                        | 9   | 9.22 ab | 6.10 bc | 119 ab  | 67.3 cd | 168 ef  |
| 1                        | 2   | 9.78 ab | 5.49 cd | 109 bcd | 59.3 d  | 170 def |
| 1                        | 10  | 7.80 ab | 5.55 cd | 117 abc | 64.7 cd | 198 abc |
| 1                        | 11  | 8.72 ab | 5.85 bc | 120 ab  | 69.8 cd | 195 a-d |

\*a-g indicate means followed by the same letter are not significantly different at the  $P < 0.05$  level according to the Duncan-Walker K-ratio t-test.

"TRT" = treatment

**Table 3**

Effect of soil treatments on soil pH and micronutrient composition of *Alyssum murale* and *Alyssum corsicum* grown on nickel-refinery contaminated Welland loam (soil 1), nickel-refinery contaminated Quarry muck (soil 2) and serpentine soils (soils 3-11) for 120 days.

| Soil   | TRT      | Final pH | Cu    | Zn    | Co    | Ni   | Mn    | Fe    |
|--|----------|----------|-------|-------|-------|------|-------|-------|
|  |          |          | mg/kg | mg/kg | mg/kg | g/kg | mg/kg | mg/kg |
| 1  |          | 5.47     | 11.0  | 156   | 136   | 8.13 | 39.2  | 67.6  |
| <u>Phosphate Series (phosphate added to the soil in kg/ha by the addition of phosphate-containing fertilizer):</u> |          |          |       |       |       |      |       |       |
| 3  | 0 P      | 5.23     | 15.0  | 179   | 99.1  | 7.58 | 56.2  | 49.6  |
| 2  | 100 P    | 5.18     | 16.0  | 131   | 102   | 7.34 | 59.7  | 50.1  |
| 4  | 250 P    | 5.24     | 14.5  | 133   | 82.2  | 7.37 | 56.8  | 56.4  |
| 5  | 500 P    | 5.13     | 14.5  | 129   | 73.8  | 6.50 | 53.1  | 50.8  |
| <u>pH Series (soil was acidified using nitric acid for "Lo pH" and "MLo pH"):</u>                                  |          |          |       |       |       |      |       |       |
| 6  | Lo pH    | 4.99     | 19.2  | 192   | 91.0  | 4.16 | 129   | 53.1  |
| 7  | MLo pH   | 5.18     | 16.8  | 160   | 104   | 5.77 | 81.2  | 64.0  |
| 2  | As is pH | 5.18     | 16.0  | 131   | 102   | 7.34 | 59.7  | 50.1  |
| 8  | Limed    | 5.57     | 10.1  | 102   | 71.1  | 9.28 | 19.9  | 57.6  |
| <u>Ca:Mg Ratio Series:</u>   |          |          |       |       |       |      |       |       |
| 9  | 1.0 Ca   | 5.25     | 17.0  | 134   | 108   | 7.32 | 65.0  | 55.0  |
| 2  | 0 Ca/Mg  | 5.18     | 16.0  | 131   | 102   | 7.34 | 59.7  | 50.1  |
| 10   | 2.5 Mg   | 5.13     | 17.4  | 152   | 90.4  | 6.75 | 48.9  | 53.0  |
| 11   | 5.0 Mg   | 5.04     | 16.2  | 149   | 87.6  | 5.71 | 54.8  | 67.1  |

"TRT" = treatment

"MLo pH" = medium-low pH

The soil designations correspond to the soil designations in Example 1.

The "pH series" experiments demonstrate that the application of limestone increases the uptake of nickel in *Alyssum* so that plant tissues accumulate an increased concentration of nickel.

***Example 3***

5           The results show an increase in the geometric mean of nickel uptake in  
plant tissue by liming *Alyssum* plants cultivated on nickel-refinery contaminated  
Quarry muck (soil 2) (Table 4) and on nickel-refinery contaminated Welloam  
loam (soil 1), nickel-refinery contaminated Quarry muck (soil 2) and selected  
10           serpentine soils (soils 3-11) (Table 5) from Example 1. The cultivation  
conditions were the same as those for Examples 1 and 2.

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**Table 4**

Effects of soil treatments on the mean concentrations of elements in whole shoots and shoot yield of *Alyssum murale* and *Alyssum corsicum* grown on nickel-refinery contaminated Quarry muck (soil 2) for 60 days.

| Soil                     | TRT | Treatment | Shoot Yld | Shoot Ni | Shoot Co | Shoot MN |
|--------------------------|-----|-----------|-----------|----------|----------|----------|
|                          |     |           | g/pot     | g/kg     | mg/kg    | mg/kg    |
| 2                        | 1   | None      | 8.46 d*   | 3.33 abc | 8.62 ab  | 27.9 bc  |
| <u>Phosphate Series:</u> |     |           |           |          |          |          |
| 2                        | 3   | 0 P       | 10.78 a-d | 3.24 bc  | 5.50 b   | 15.0 bc  |
| 2                        | 2   | 100 P     | 12.09 a   | 3.23 bc  | 5.75 ab  | 14.5 bc  |
| 2                        | 4   | 250 P     | 11.53 abc | 3.76 a   | 5.50 b   | 18.6 bc  |
| 2                        | 2   | 500 P     | 11.86 ab  | 3.30 abc | 6.38 ab  | 27.7 bc  |
| <u>pH Series:</u>        |     |           |           |          |          |          |
| 2                        | 6   | Lo pH     | 12.01 ab  | 1.48 e   | 10.25 a  | 59.8 a   |
| 2                        | 7   | Med pH    | 9.44 bcd  | 2.12 d   | 6.12 ab  | 29.0 b   |
| 2                        | 2   | As is pH  | 12.09 a   | 3.23 bc  | 5.75 ab  | 14.5 bc  |
| 2                        | 8   | Limed     | 11.14 abc | 3.72 ab  | 5.88 ab  | 13.3 c   |
| <u>Ca:Mg Series:</u>     |     |           |           |          |          |          |
| 2                        | 9   | Ca        | 9.08 cd   | 3.42 abc | 6.38 ab  | 16.3 bc  |
| 2                        | 2   | As is Ca  | 12.09 a   | 3.23 bc  | 5.75 ab  | 14.5 bc  |
| 2                        | 10  | Med Mg    | 11.66 ab  | 3.03 c   | 4.62 b   | 24.9 bc  |
| 2                        | 11  | Hi Mg     | 9.98 a-d  | 2.94 c   | 5.25 b   | 23.3 bc  |

\*a-e indicate means followed by the same letter are not significantly different at the  $P < 0.05$  level according to the Duncan-Walker K-ratio t-test.

"TRT" = treatment

**Table 5**

Effect of altering nickel-refinery contaminated Welland loam (soil 1), nickel-refinery contaminated Quarry muck (soil 2) and serpentine soils (soils 3-11) by adding phosphate, adjusting the pH or adjusting the Ca:Mg ratio on soil pH, mean yield and micronutrient composition of shoots of *Alyssum* species grown for 120 days (GM designates geometric mean).

| Soil   | TRT      | Final pH | GM-Yield | GM-Ni | GM-Co | GM-Mn | GM-Zn | GM-Fe | GM-Cu |
|--|----------|----------|----------|-------|-------|-------|-------|-------|-------|
|  |          |          | g/pot    | mg/kg |       |       |       |       |       |
| 1  | None     | 6.34     | 20.2     | 5460  | 7.6   | 11.9  | 151   | 61    | 4.8   |
| <u>Phosphate Treatments (phosphate added to the soil in kg/ha by the addition of phosphate-containing fertilizer):</u> |          |          |          |       |       |       |       |       |       |
| 3  | 0 P      | 6.09     | 41.6     | 4400  | 5.8   | 16.5  | 152   | 56    | 4.2   |
| 2  | 100 P    | 6.05     | 42.7     | 4120  | 5.7   | 18.6  | 126   | 57    | 4.5   |
| 4  | 250 P    | 6.07     | 49.9     | 4120  | 5.1   | 21.4  | 143   | 57    | 4.8   |
| 5  | 500 P    | 5.98     | 46.4     | 3800  | 5.1   | 22.9  | 139   | 54    | 4.2   |
| <u>pH Treatments (soil was acidified using nitric acid for "Lo pH" and "Med-pH"):</u>                                  |          |          |          |       |       |       |       |       |       |
| 6  | Lo pH    | 5.44     | 32.2     | 2010  | 6.8   | 50.5  | 153   | 68    | 6.4   |
| 7  | Med-pH   | 5.76     | 36.1     | 2700  | 4.5   | 21.0  | 143   | 60    | 4.8   |
| 2  | As is pH | 6.05     | 42.7     | 4120  | 5.7   | 18.6  | 126   | 57    | 4.5   |
| 8  | Limed    | 6.20     | 40.5     | 4520  | 6.3   | 15.8  | 137   | 55    | 4.1   |
| <u>Ca:Mg Treatments:</u>   |          |          |          |       |       |       |       |       |       |
| 9  | 0.0 Ca   | 6.13     | 38.6     | 4510  | 6.3   | 16.2  | 135   | 57    | 4.8   |
| 2  | 1.0 Ca   | 6.05     | 42.7     | 4120  | 5.7   | 18.6  | 126   | 56    | 4.5   |
| 10   | 2.5 Mg   | 5.98     | 39.0     | 4410  | 5.9   | 16.2  | 146   | 63    | 4.6   |
| 11   | 5.0 Mg   | 5.91     | 44.0     | 4260  | 5.8   | 18.3  | 158   | 58    | 4.6   |

"TRT" = treatment

The soil designations correspond to the soil designations in Example 1.

**Example 4 - Novel Hyperaccumulators**

The concentration of elements in the shoots of *Alyssum* species grown on a field of serpentine colluvial soil in Josephine County, Oregon, are shown in Table 6 below.

**Table 6**

| Row | Species            | Genotype | Block | Zn  | P    | Cu | Co | Ni    | Mn | Fe   | Mg   | Ca   | K    |
|-----|--------------------|----------|-------|-----|------|----|----|-------|----|------|------|------|------|
| 139 | <i>A. corsicum</i> | 16       | 1     | 137 | 5.01 | 9  | 14 | 13400 | 53 | 53.8 | 5.52 | 20.9 | 43.3 |
| 483 | <i>A. corsicum</i> | 16       | 2     | 141 | 4.08 | 8  | 16 | 17500 | 32 | 755  | 5.99 | 24.2 | 44.3 |
| 129 | <i>A. murale</i>   | 49       | 1     | 99  | 4.80 | 7  | 12 | 14100 | 41 | 397  | 3.98 | 32.1 | 41.4 |
| 325 | <i>A. murale</i>   | 49       | 2     | 106 | 4.63 | 8  | 16 | 17100 | 46 | 455  | 5.32 | 31.7 | 41.7 |
| 135 | <i>A. murale</i>   | 54       | 1     | 119 | 4.18 | 5  | 13 | 15600 | 53 | 927  | 4.02 | 25.8 | 44.5 |
| 143 | <i>A. murale</i>   | 69       | 1     | 165 | 5.78 | 5  | 16 | 16700 | 53 | 380  | 4.52 | 17.3 | 38.8 |
| 553 | <i>A. murale</i>   | 69       | 2     | 191 | 4.97 | 6  | 15 | 13400 | 45 | 616  | 5.66 | 25.4 | 6.16 |

The elements are present in mg/kg amounts.

Whole shoots or side branch samples containing stems and leaves were collected from pots or the field for each genotype, dried in forced air drying ovens and ground with a non-contaminating mill to less than about 0.1 mm. The ground samples were then placed in a borosilicate beaker and ashed at 480°C overnight. Nitric acid was added to dissolve the resultant ash which was then heated until dry on a hot plate. Hydrochloric acid (3.0 M) was added and the beaker was refluxed for two hours to determine recovered nickel concentration. Concentrations of nickel were measured by an inductively coupled argon plasma emission spectrometer. Low concentrations were measured by atomic absorption spectrometry.

This invention has been described in specific detail with regard to specific plants and methods for increasing metal, such as nickel, uptake via phytomining or phytoextraction. Except where necessary for operability, no limitation to these



5 specific materials is intended nor should such a limitation be imposed on the claims appended hereto. From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions without undue experimentation. All patents, patent applications and publications cited herein are incorporated by reference in their entirety.

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***What Is Claimed Is:***

1. A method for selectively increasing the amount of at least one metal recovered from metal-containing soil comprising:

- (a) elevating the pH of the soil; and
- (b) cultivating at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate said at least one metal from the soil in above-ground tissue.

2. The method of claim 1, wherein said at least one metal is nickel.

3. The method of claim 1, wherein the pH of the soil is elevated by adding to the soil at least one agent that results in an increase in the soil pH.

4. The method of claim 3, wherein the at least one agent that results in an increase in the soil pH is selected from the group consisting of limestone, dolomitic limestone, lime, hydrated lime, limestone equivalents and mixtures thereof.

5. The method of claim 1, further comprising after said cultivating:

- (c) lowering the pH of the soil; and
- (d) cultivating the at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one second metal from the soil in above-ground tissue.

6. The method of claim 5, wherein the at least one second metal is selected from the group consisting of: cobalt, palladium, rhodium, ruthenium, platinum, iridium, osmium, rhenium and mixtures thereof.

7. The method of claim 5, wherein the pH of the soil is lowered by adding to the soil at least one agent that results in a decrease of the soil pH.
8. The method of claim 1, wherein said at least one plant is an *Alyssum* plant.
- 5 9. The method of claim 8, wherein said *Alyssum* plant is selected from the group consisting of: *A. murale*, *A. pintodasilvae*, *A. serpyllifolium*, *A. malacitanum*, *A. lesbiacum*, *A. fallacinum*, *A. argenteum*, *A. bertolonii*, *A. tenium*, *A. heldreichii*, *A. corsicum*, *A. pterocarpum*, *A. caricum* and mixtures thereof.
- 10 ~~10.~~ A method for recovering nickel from nickel-containing soil comprising:
  - (a) elevating the pH of the soil;
  - (b) cultivating at least one nickel-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel;
  - 15 (c) harvesting said at least one plant; and
  - (d) recovering nickel from said harvested plant.
11. The method of claim 10, wherein the soil has a calcium concentration of between about 20-80%.
- 20 12. The method of claim 10, wherein the nickel is recovered by drying and combusting the harvested plant to oxidize and vaporize organic material present.
13. The method of claim 10, wherein said at least one plant is an *Alyssum* plant.

14. The method of claim 13, wherein said *Alyssum* plant is selected from the group consisting of: *A. murale*, *A. pintodasilvae*, *A. serpyllifolium*, *A. malacitanum*, *A. lesbiacum*, *A. fallacinum*, *A. argenteum*, *A. bertolonii*, *A. tenium*, *A. heldreichii*, *A. corsicum*, *A. pterocarpum*, *A. caricum* and mixtures thereof.

15. The method of claim 14, wherein said *Alyssum* plant is selected from the group consisting of: *A. corsicum* G16, *A. murale* G69, *A. murale* G82 and mixtures thereof.

16. The method of claim 10, wherein at least about 2.5% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel.

17. The method of claim 16, wherein at least about 3.0% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel.

18. The method of claim 17, wherein about 4.0% of the above-ground tissue of said at least one plant, on a dry weight basis, is nickel.

19. A method for recovering cobalt from cobalt-containing soil comprising:  
(a) lowering the pH of the soil;  
(b) cultivating at least one cobalt-hyperaccumulator plant in the soil under conditions such that at least 0.1% of the above-ground tissue of said at least one plant, on a dry weight basis, is cobalt;  
(c) harvesting said at least one plant; and  
(d) recovering cobalt from said harvested plant.

20. The method of claim 19, wherein the soil has a calcium concentration of between about 20-80%.

21. The method of claim 20, wherein said at least one plant is an *Alyssum* plant.
22. The method of claim 21, wherein said *Alyssum* plant is selected from the group consisting of: *A. murale*, *A. pintodasilvae*, *A. serpyllifolium*, *A. malacitanum*, *A. lesbiacum*, *A. fallacinum*, *A. argenteum*, *A. bertolonii*, *A. tenium*, *A. heldreichii*, *A. corsicum*, *A. pterocarpum*, *A. caricum* and mixtures thereof.
23. An isolated *Alyssum* plant species that accumulates nickel in quantities greater than that accumulated by *A. murale* 103.
24. An isolated *Alyssum* plant species cultivated on nickel-containing soil that accumulates nickel in above-ground tissue at a concentration of 1.55% or greater by weight based on the gross dry weight of said tissue.
25. The isolated *Alyssum* plant species of claim 23 or 24, wherein the species is *A. corsicum* G16.
26. The isolated *Alyssum* plant species of claim 23 or 24, wherein the species is *A. murale* G69.
27. The isolated *Alyssum* plant species of claim 23 or 24, wherein the species is *A. murale* G82.
28. Pollen of the plant of claim 23 or 24.
29. A plant having all the physiological and morphological characteristics of the plant of claim 23 or 24.

30. Propagation material of the plant of claim 23 or 24.

~~31.~~ An *Alyssum corsicum* G16 seed as deposited with the American Type Culture Collection under accession number 203436.

~~32.~~ An *Alyssum murale* G69 seed as deposited with the American Type Culture Collection under accession number 203437.

~~33.~~ An *Alyssum murale* G82 seed as deposited with the American Type Culture Collection under accession number 203438.

34. A plant produced by growing the seed of any one of claims 31-33.

35. Pollen of the plant of claim 34.

36. A plant having all the physiological and morphological characteristics of the plant of claim 34.

37. Propagation material of the plant of claim 34.

~~38.~~ A method for decontaminating metal-containing soil, comprising cultivating at least one hyperaccumulator plant in metal-containing soil, whereby the concentration of metal in the above-ground plant tissue of said at least one hyperaccumulator plant exceeds the concentration of metal in said soil by a factor of at least 2.

39. The method of claim 38, wherein the at least one hyperaccumulator plant exceeds the concentration of metal in said soil by a factor of 3.

40. The method of claim 39, wherein the at least one hyperaccumulator plant exceeds the concentration of metal in said soil by a factor of 4.

41. A method for selectively increasing the amount of at least one metal recovered from metal-containing soil comprising:

- (a) lowering the pH of the soil; and
- (b) cultivating at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate said at least one metal from the soil in above-ground tissue.

42. The method of claim 41, wherein said at least one metal is selected from the group consisting of: cobalt, palladium, rhodium, ruthenium, platinum, iridium, osmium, rhenium and mixtures thereof.

43. The method of claim 41, wherein the pH of the soil is lowered by adding to the soil at least one agent that results in a decrease of the soil pH.

44. The method of claim 41, further comprising after said cultivating:

- (c) raising the pH of the soil; and
- (d) cultivating the at least one metal-hyperaccumulator plant in the soil under conditions sufficient to permit said at least one plant to accumulate at least one second metal from the soil in above-ground tissue.

45. The method of claim 44, wherein the pH of the soil is elevated by adding to the soil at least one agent that results in an increase in the soil pH.

46. The method of claim 45, wherein the at least one agent that results in an increase in the soil pH is selected from the group consisting of limestone, dolomitic limestone, lime, hydrated lime, limestone equivalents and mixtures thereof.

47. The method of claim 44, wherein the at least one second metal is nickel.

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## Recovering Metals from Soil

### *Abstract*

5 The invention relates to recovering metals, such as nickel and cobalt, by phytomining or phytoextracting soils rich in metals wherein the desired metal is selectively accumulated in hyperaccumulator plants by adjusting the soil pH. The metals are ultimately recovered from above-ground plant tissues at economically acceptable levels without further contaminating the metal-containing sites. The invention also relates to metal-hyperaccumulating plants.